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The Determination of Calcium in Serum by Flame Photometry

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Since 1948, when Riehm first demonstrated the feasibility of the direct flame-photometric method for the determination of calcium in dilute solutions, this principle has been applied to analysis of soil (Stanford & English, 1949), milk (Keirs & Speck, 1950) and serum (Severinghaus & Farabee, 1950). In the following year further reports of serum calcium determinations appeared from Leyton (1951) and from Mosher, Itano, Boyle, Myers & Iseri (1951), followed by those from Kapuscinski, Moss, Zak & Boyle (1952), Zak, Mosher & Boyle (1953), and Powell (1953). While the present account was being written, direct determinations on diluted serum have been described by Winer & Kubins (1953), Chen & Toribara (1953) and by McIntyre (1954). Several of the American authors used modified spectrophotometers, and in nearly all cases sera have been prepared for analysis by procedures involving oxidation, precipitation of calcium, or removal of sodium. The comparative advantages of simply diluting with water are evident. Although many forms of photometer, most of them elaborate, have been described, the desirability of using simple and relatively inexpensive equipment still remains.

The determination of calcium in blood by flame photometry presents peculiar difficulties. The concentration of calcium is small but that of sodium and potassium large, while the concentration range of calcium occurring in human sera is narrow, so that considerable accuracy is required. The normal range in human serum is 9·0–11·5 mg. Ca/100 ml., and even grossly pathological conditions extend this ambit only to the limits 4–16 mg./100 ml. If precipitation and other pretreatment of the sample are to be avoided, the serum must be diluted at least tenfold, so that the final concentration of calcium is about 1 mg./100 ml. This dilute calcium solution emits very little light from a flame. The use of an interference filter permits the partial isolation of the calcium oxide red bands (approx. 6200 Å), provided that didymium glass is used to reduce the excess of sodium light also emitted. In an acetylene–air flame, maximum output of energy in the calcium bands is ensured, and the intensity of this light, although very low, can be measured either by the use of a photoconductive cell (Schwarz, 1948) as described below, or by means of photomultiplier apparatus.

The construction and use of an internal standard photometer which has proved satisfactory for the determinations of calcium, sodium and potassium in diluted serum and other material are described below.

EXPERIMENTAL

Description of photometer

The general lay-out of the instrument is shown diagrammatically in Fig. 1.

The burner unit. Acetylene is burnt after admixture with the air-borne sample, and no atmospheric air enters the system. The brass Meker-type burner is shown in section in Fig. 2. The jet (*A*) is a standard welding component (British Oxygen Co. Ltd., London, type DH, 2 cu.ft./hr. i.e. 940 ml./min.), and is screwed into position over a fibre washer (*B*). The top-plate (*C*), of brass, is perforated as shown. Acetylene at a pressure only slightly above atmospheric enters at *D* and the air-mist through *E*. The burner is clamped in a boss attached to a plate (*A*, Fig. 3) bearing a light-trap (*B*) and bolted to a plate (*C*) of similar outline brazed to the chimney-tube (*D*) which it supports. This tube has diametrically opposed apertures (*E*, *E'*) for exit of light, and a smaller hole (*F*) for insertion of a taper for lighting.

Gas control and atomizer. Both acetylene and air are taken from industrial cylinders fitted with two-stage regulators. The acetylene regulator is fitted with an outlet needle-valve which is used as on-off control to avoid readjustment of the succeeding valve. Fine needle-valves incorporated in the photometer are used for both gases. Acetylene from its

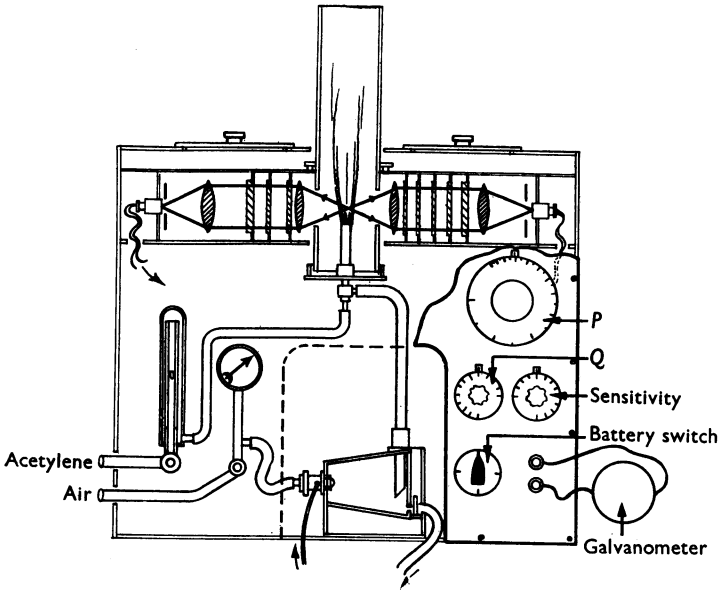

Fig. 1. General arrangement of photometer. For significance of *P* and *Q* see Fig. 7.

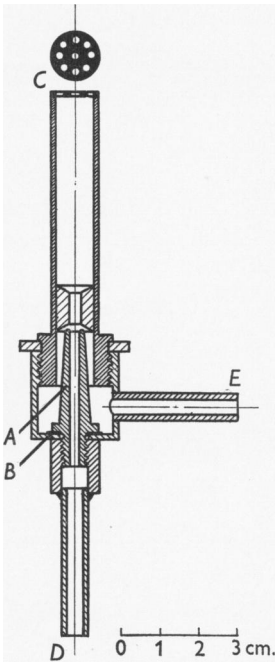

Fig. 2. Details of burner.

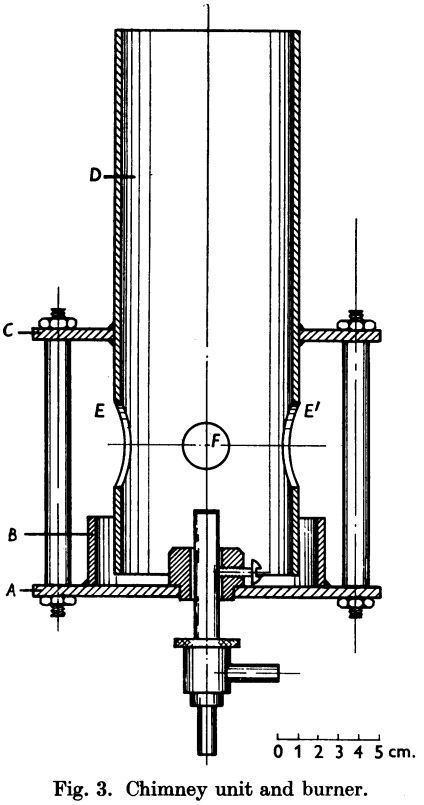

Fig. 3. Chimney unit and burner.

control-valve passes through a flow-meter, a stock component (Rotameter Manufacturing Co. Ltd., Croydon), calibrated in c.c. *cyclo*propane/min. The air supply, adjusted similarly to a pressure of 15 lb./sq.in. (1·06 kg./sq.cm.) read from a gauge fitted by a T-joint to the outflow tube, is led to the spray. This latter component (Fig. 4), derived from a basic design by Wright (private communication, 1951), is of concentric form with the gas-orifice in the centre and is made from Perspex, with an ebonite plug (*A*) to which a small vinyl plastic tube is fitted for admission of the sample. With air at 15 lb./sq.in., good operation is obtained with flow-rates varying from 1 to 25 ml. water/min. The spray is fitted into an aperture (*A*, Fig. 5) in a chamber of Perspex, so that the mist entering the chamber strikes the back of the outlet tube (*B*, Fig. 5), allowing removal of large droplets and the issue of a stably suspended sample to the burner. The condensate drains through a trap as shown.

Optical system. As indicated in Fig. 1, this is symmetrical apart from different filters. Light, rendered parallel, passes through heat filters (Chance Bros. Ltd., Birmingham, ON 20) and then through filters which transmit Li light on one side and Ca light on the other. The beams finally converge on the apertures of the photocells. The latter are Schwarz photoconductive cells (Hilger & Watts, Ltd., London, F.T. 404), protected by shutters. The filters for Ca light are Chance OY 1, ON 16 and an interference filter (peak 6200 Å, Barr and Stroud, Ltd., Glasgow). For lithium, a Chance OR 1 and an interference filter (peak 6700 Å) are used. All are of 2 in. diameter. The Chance glasses are 2 mm. thick, except for the ON 16 (3 mm.). Examination with a spectrophotometer of the combinations of filters selected for use in the several determinations gave the results shown in Fig. 6, in which the wavebands used are indicated.

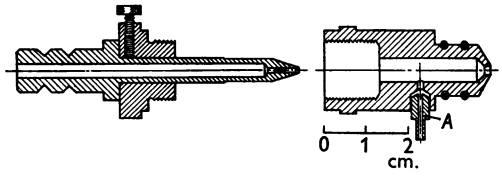

Fig. 4. Details of spray.

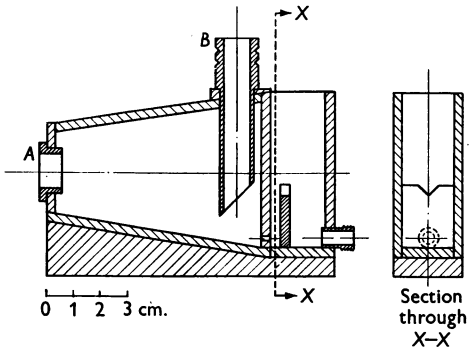

Fig. 5. The spray chamber.

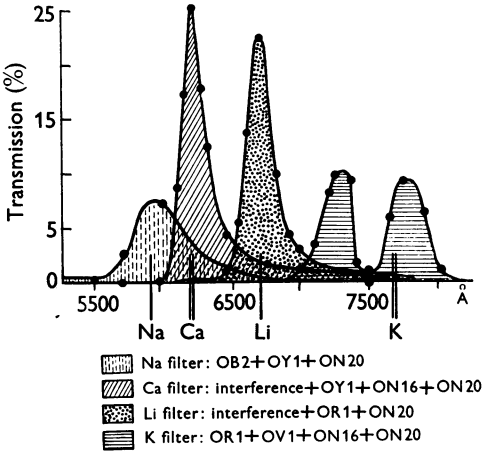

Fig. 6. Characteristics of filter combinations.

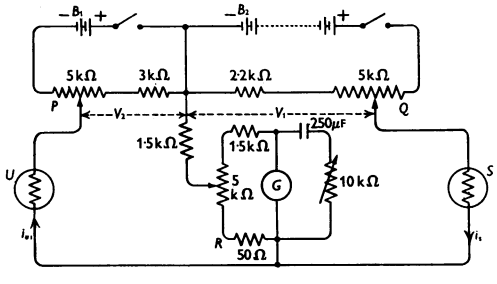

Fig. 7. Electrical circuit.

Electrical system. The photocells are matched for dark-current, fatigue and sensitivity. In the dark they have resistances of 8 MΩ or more; in operation they are equivalent to resistances of the order of 1·5–0·5 MΩ. The electrical circuit is illustrated in Fig. 7. A fraction, V_1, of the potential from one battery (determined by the potentiometer Q) causes current (i_s) through the cell S to flow through the galvanometer (G) in one direction, and potential V_2 derived from a second battery is adjusted until the current (i_u) through the cell U, passing through G in the opposite direction, balances i_s. Choice of potentials depends on the characteristics of the individual photocells: values in the present instrument are quoted for guidance. For determination of Ca, 3 v are used (B_1, Fig. 7) while B_2 is of 30 v. Cell S responds to light from the lithium and i_s during operation is made equal to the value of i_u that is obtained on spraying a standard solution of unknown or mid-range concentration. When i_s has thus been set up it is left unchanged for the course of the series of measurements. For each unknown solution sprayed subsequently, the setting of P in Fig. 7 is adjusted for null-point, and readings from a dial on this potentiometer (engraved with a reciprocal scale) indicate the concentrations of unknown.

The galvanometer (450 Ω, 1 μA full-scale deflexion, Cambridge Instrument Co. Ltd., London) is damped by a capacity–resistance circuit, and is operated at any sensitivity in the range 1·5–150 μA full-scale deflexion by adjustment of the shunt (R, Fig. 7). Alteration of the sensitivity thus has no effect on the balance-point or on damping.

Method for determination of serum calcium with internal standard

Serum (1 ml.) is diluted with 5 ml. of 0·2 mM-Li_2SO_4 and 4 ml. of water. The standard solutions have the composition NaCl, 14·3 mM; KCl, 0·513 mM; Li_2SO_4, 0·100 mM; Ca (as $CaCl_2$), 0·75, 1·0 or 1·25 mg./100 ml. They are made from a common concentrated stock solution containing NaCl, KCl and Li_2SO_4; samples of this solution are mixed with appropriate volumes of stock $CaCl_2$, made from $CaCO_3$ and HCl, and brought to uniform volumes. These solutions are all kept in polythene bottles.

The spray is adjusted to consume 4–9 ml. water/min. Acetylene is turned on to show 800–900 units on the scale of the flowmeter, and the flame is lit. The power supply is switched on and the cell shutters opened. With galvanometer sensitivity about 50 % and the dial of P (Figs. 1 and 7) set at any convenient mark at the low end of its scale (maximum potential), the low standard (0·75 mg. Ca/100 ml., corresponding to a 1 to 10 dilution of serum containing 7·5 mg. Ca/100 ml.), is sprayed and Q (Figs. 1 and 7) is adjusted for null-point. The medium standard is then sprayed and the sensitivity is adjusted so that the out-of-balance current in the galvanometer is equivalent to 20–30 mm. deflexion. The setting of P for null-point is found; the high standard is next sprayed and the reading on P at balance is also recorded. A second series of readings with the standards allows minor adjustments to be made and provides data for the calibration graph. The diluted serum is now sprayed and the Ca content deduced from this graph. Several determinations can be made when the photometer has been set up, and the calibration checked at the end by repeating the standard readings. Minor drift in calibration is additive in nature and can be corrected by adjustment of Q

to retain balance at the original setting of P when one of the standard solutions is sprayed.

Method for determination of serum sodium and potassium with internal standard

With suitable filters, the instrument described above has been used satisfactorily for the determination of sodium and potassium. For sodium, the filters (Chance) are OY1 and OB2; and for potassium (7700 Å), OR1 and OV1. For either determination it was found convenient to use 1 in 101 dilutions of serum: for determination of sodium, serum (0·2 ml.) is diluted by addition to 20 ml. of 0·25 mM-Li_2SO_4 solution, while for determination of potassium 0·2 ml. of serum is added to a mixture of 10 ml. 2·2 mM-Li_2SO_4 with 10 ml. of 0·1 % (w/v) NaCl, the latter being included because of the enhancing effect of sodium on the emission of potassium (Domingo & Klyne, 1949). The procedure for determination of sodium and potassium is analogous to that described for calcium.

RESULTS

Single-cell measurement of calcium. Although in applications of the photometer the internal-standard method is employed, single-cell measurements were used as an aid in development and in exploring the operating conditions.

Comparison of the responses of a single photocell to calcium showed that acetylene produced about 30 times the energy of coal-gas. Use of oxygen and air with the coal-gas did not increase the response significantly above that found with air only. When a barrier-layer cell was used, with an acetylene flame, sensitivity was still far lower than the minimum required for analysis of serum. With the Schwarz photocell, aqueous calcium chloride (1 mg. Ca/100 ml.) gave a current of 0·31 μA, corrected for water-blank (0·015 μA). The dark-current and flame background-current were imperceptible. A solution of potassium chloride (2 mg. K/100 ml.) gave 0·009 μA (corrected) and a sodium chloride solution (30 mg. Na/100 ml.) gave even less. Lithium sulphate (0·1 mM) gave a corrected current in the 'calcium' cell of 6 % of that found with the calcium. Cell-currents bore linear relationships to the concentrations of calcium sprayed. Variations of the air-pressure of the order of ± 20 % caused only small changes in the rate of consumption of sample but resultant cell-currents varied by ± 15 %. When the acetylene flow was changed, the effect was a little greater. With optimum values for air-pressure, spray-setting and acetylene-flow, repeated spraying of a calcium sample gave a deflexion with no significant drift, but which showed short-term (1–2 sec.) irregular variations within the range + 10 to − 5 %.

The use of lithium. In order to determine the correct concentration of lithium to be used as an internal standard, preliminary experiments were carried out with solutions of pure lithium sulphate, using the 'lithium' photocell. Under these conditions the potential (B_2, in Fig. 7) was then chosen so that the rate of increase in cell current with increased lithium concentration matched the corresponding relationship for calcium (measured with the 'calcium' cell). With the battery potential thus adjusted, a standard concentration for lithium (10^{-4} M) was then found so that the 'lithium current' approximately equalled the 'calcium current' for a concentration of calcium midway in the working range. The effects of calcium, sodium, potassium, phosphate and water on the 'lithium' photocell were all imperceptible. It was found, again by single-cell measurements, that variations in the flow rate of acetylene affected the responses to calcium and lithium as shown in Fig. 8, from which the rate for optimum stability can be deduced. At this optimum rate, changes in air-pressure led to only negligible changes in response.

When the photometer was used with lithium internal standard in the optimum conditions found as described above, the stability was greater than under single-cell conditions; it was possible therefore to use higher galvanometer sensitivity. Decrease in air pressure by 30 % did not affect the accuracy of determinations, nor did ± 15 % change in acetylene flow. There was no discernible long-term change in the value of a given calcium solution when determined by simultaneous use of calcium standards, lithium being the reference-substance.

Table 1. *Estimation of* Ca *in inorganic solutions by photometric and titration methods*

The solutions contained Na and K equivalent to serum diluted 1 to 10.

	Concentrations found			
	Photometer		Titration	
Theoretical concentration (mg. Ca/l.)	(mg. Ca/l.)	Error (%)	(mg. Ca/l.)	Error (%)
9·0	8·9	− 1·1	9·5	+ 5·5
9·5	9·3	− 1·9	9·7	+ 1·9
10·6	10·6	0	11·2	+ 5·3
11·1	11·6	+ 4·5	11·4	+ 2·7
11·7	11·8	+ 0·9	12·2	+ 4·3

Table 2. Ca *content of different sera determined by photometric and titration methods*

Method	Ca content (mg./l.)								
Photometric	8·3	9·7	9·7	9·9	9·9	10·3	10·4	10·5	10·5
Titration	8·9	9·8	9·8	10·4	9·7	10·0	10·2	10·3	10·8

Table 3. *Recovery of* Ca *added to serum*

Results expressed as mg. Ca/100 ml. of undiluted sample.

Ca added	Ca found	Added Ca recovered	Ca added	Ca found	Added Ca recovered
0	9·9	0	4·2	14·4	4·5
1·1	10·9	1·0	9·1	18·5	8·6
2·1	12·0	2·1	13·7	23·7	13·8
3·2	13·0	3·1	18·2	28·1	18·2

Table 4. *Effect of variations in* K, Na *and phosphate contents of sample on apparent* Ca *concentration*

Samples diluted 1 to 10.

(i) Na = 330 mg./100 ml. True Ca = 10 mg./100 ml.

K in sample (mg./100 ml.)	6	12	18	24	30
Ca found (mg./100 ml.)	11·9	10·9	10·2	9·4	8·9

(ii) K = 20 mg./100 ml. True Ca = 10 mg./100 ml.

Na in sample (mg./100 ml.)	198	264	333	462
Ca found (mg./100 ml.)	9·6	9·9	10·0	10·3

(iii) Na = 330 mg./100 ml.; K = 20 mg./100 ml. True Ca = 11·6 mg./100 ml.

P in sample (mg./100 ml.)	2	4	5	8	10
Ca found (mg. Ca/100 ml.) (average of 3)	11·5	11·3	11·0	10·9	11·1

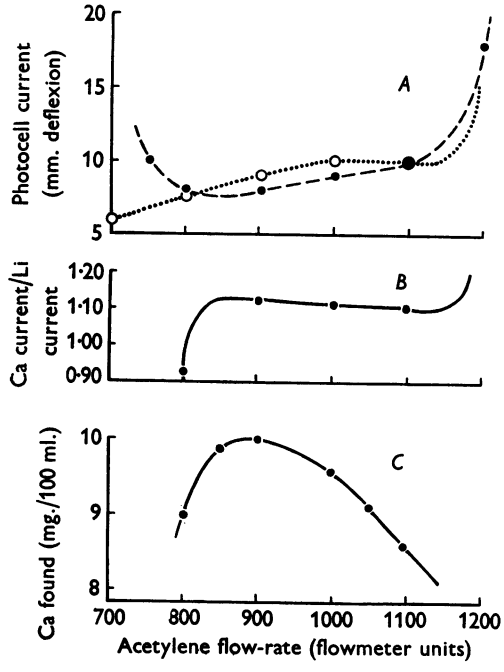

Fig. 8. Effects of acetylene flow-rate on (*A*), single-cell currents for Ca (. . . .) and Li (– – –); (*B*), ratio of Ca to Li currents above; (*C*) readings by internal standard. True Ca = 10 mg. %, diluted 1 to 10, with Na and K as in standards. Spray rate = 5·2 ml./min.

Nor did the calibration curve alter throughout a 1·5 hr. trial by more than enough to confer an error of ± 5 %, while over shorter times the error was negligible.

Use of lithium for additive cancelling out of background effects (Leppänen, Krusius & Mettinen, 1952), proved better than single-cell working, but inferior to the proportional null-point adjustment described above.

Calcium determinations. The accuracy of internal-standard determinations of calcium was tested first with 'unknown' inorganic solutions which were also analysed by a standard oxalate method (with ceric sulphate titration, Vogel, 1948). Results are shown in Table 1. Sera were then analysed by the same procedures, with the results shown in Table 2.

The results shown in Table 3 indicate that satisfactory recoveries of calcium added to serum are obtained by the internal-standard method.

Interference effects. Inaccuracies due to variations in concentrations of sodium, potassium and phosphate were studied, the range in each case being that corresponding to the widest values expected in pathological sera. Whereas increase in sodium raised the apparent concentration of calcium, increased potassium had the opposite effect. As seen from Table 4, errors in the measurement of serum calcium do not exceed 5 %, even in the most adverse clinical conditions, and can be corrected if

values for sodium and potassium are known. The effect of phosphate is also small.

The introduction of 16% (v/v) *iso*propanol in the diluted samples increased the single-cell responses by 70% (see Alkemade, Smith & Verschure, 1951), but conferred no advantage in the internal-standard method. Fig. 8 shows the effect of acetylene flow-rate on calcium determinations made with the single cell and the internal standard methods.

DISCUSSION

The photometric determinations described give results which agree well with those obtained titrimetrically, and which show an average difference from theoretical of less than ±2% (Table 1). They are obtained rapidly and without elaborate preparation of the sample; measurements of serum calcium by the internal-standard method are much to be preferred to those made with a single cell.

Although it is clear that use of an internal standard enhances stability and reproducibility (Spencer, 1950; Bernstein, 1952), it is essential to ensure that the conditions in which it is used are optimum. Failing this, use of the method may introduce errors greater than those occurring in single-cell determinations (Fig. 8).

Of the bands in the calcium flame-spectrum, that at 6200 Å is detected most sensitively by the photoconductive cells, whose characteristic curve (Schwarz, 1950) shows a peak at 7200 Å. With given illumination these cells pass current proportional to the potential applied, which may be up to 100 v. Thus the sensitivities of a pair of cells may be matched easily by choice of potentials. The ultimate sensitivity (Schwarz, 1951) is of the same order as that of a photomultiplier tube.

SUMMARY

An internal standard flame photometer for estimation of calcium, sodium and potassium in serum, with dilution as the only pretreatment of the sample, is described, and some results are reported.

The author thanks Professor R. H. S. Thompson for continued interest and encouragement, Dr B. M. Wright (Medical Research Council Pneumoconiosis Unit, Penarth, Glamorganshire) for information generously given on sprays, Dr E. Schwarz (Messrs Hilger and Watts), who made the photocells to meet the present requirements and advised on their properties, and Mr G. Clough of this Department, who carried out titrimetric estimations of calcium.

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Reactions of Free Haematins and Haemoproteins with Nitric Oxide and certain other Substances

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The reversible combination of nitric oxide with haemoglobin was discovered in 1865 by Hermann, who observed the changes not only in the colour but also in the absorption spectrum of a solution of haemoglobin treated with nitric oxide in the complete absence of oxygen. By careful gasometric experiments he showed that haemoglobin combines with the same amount of nitric oxide as of carbon monoxide and that the affinity of haemoglobin for nitric oxide was greater than for carbon monoxide, and therefore much greater than for oxygen.

It was claimed by Linossier (1887) that free haematin also combines with nitric oxide. This reaction, which, as he mentions himself, was discovered by his students, was not clearly described

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